REMARKS

The Official Action of July 18, 2008, made final and the references cited therein have been carefully considered. The Applicant respectfully requests reconsideration of the application in view of the following remarks.

No claims have been amended. Claims 51-60 are pending in the application.

I. Rejection of Claims 351-60 under 35 U.S.C. § 112, First Paragraph

Claims 51-60 stand rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. Applicants respectfully traverse this rejection and submit that, in view of the specification and the state of the art, one of ordinary skill in the art could practice the claimed invention without undue experimentation.

In rebuttal to the Examiner's response, Applicants respectfully submit that one of ordinary skill in the art would be able to employ a a T-type calcium channel antagonists having the specified selectivity and potency, for reducing the number of awakenings during sleep without undue experimentation. Applicants' claims are not directed to particular compounds per se, but to the use of compounds having specific, ascertainable properties for effecting a specific, ascertainable therapeutic outcome.

In rebuttal to the Examiner's response, Applicants respectfully submit that in June 2001, if not earlier (i.e. before to the priority date of the present application), mibefradil was known to potentially inhibit many other ion channels, including L-type calcium channels.

Further to Applicants' submission of Lory, et al. (Expert Opin. Ther. Targets (2007), 11(5) 717-722) in their previous response, Applicants are submitting herewith a copy of the reference that is cited in Footnote #18 of Lory, et al. This reference (Leuranguer, V., et al., Journal of Cardiovascular Pharmacology, 37 (6), 649-661, Published: June 2001, "Inhibition of T-type and L-type calcium channels by mibefradil: Physiologic and pharmacologic bases of cardiovascular effects.") teaches that mibefradil is a potent blocker of L-type calcium channels and that the previously-held belief that mibefradil is a selective T-type antagonist is incorrect (see, e.g. Abstract and pg. 660, column 1, second paragraph).

With respect to the Nature of the Invention, the present claims are directed to a method for "reducing the number of awakenings during sleep" in a mammalian patient by using a T-type calcium channel antagonists that have a specified selectivity and potency, i.e. selectivity for the T-type calcium channel relative to the L-type calcium channel of at least 100 fold, selectivity for the α1I subtype T-type calcium channel relative to the α1G subtype T-type calcium channel of at least 10 fold, selectivity for the α1I subtype T-type calcium channel relative to the α1H subtype T-type calcium channel of at least 10 fold, and potency of an IC_{50} for binding to the T-type calcium channel of 500 nM or less. Accordingly, the nature of the invention is that it is directed to the use of compounds having specific, ascertainable properties for effecting a specific, ascertainable therapeutic outcome.

With respect to the Breadth of the Claims, Applicants note that the present invention is not directed to particular compounds per se. The present claims are directed to the use of compounds having specific, ascertainable properties for effecting a specific, ascertainable therapeutic outcome.

With respect to Guidance of the Specification and Working Examples, Applicants note that the claims are directed to the use of T-type calcium channel antagonists having a specified selectivity for reducing the number of awakenings during sleep in a mammalian patient. In this regard, the preclinical data in Example 2 demonstrates that T-type calcium channel antagonists reduce the number of awakenings during sleep relative to vehicle.

The Examiner previously indicated that no structures were provided for the compounds A-C in the table on page 17. Applicants note that although these compounds vary widely in their chemical structures, they have a common ability to selectively inhibit T-type calcium channel function and be useful in enhancing sleep. Applicants note that these compounds are more fully described on page 19 of their provisional application USSN 60/419,203, filed October 17, 2002:

Compound B: 2-(R)-(1-(R)-(3,5-bis(trifluoromethyl)phenyl)ethoxy)4-(5-(dimethyl-amino)methyl-1,2,3-triazol-4-yl)methyl-3-(S)-(4-fluorophenyl)morpholine (US 5,612,337)

T-type IC50	L-type IC50
~50 nM	> ~700nM

Compound C: 2-methoxy-5-(5-trifluoromethyl-tetrazol-1-yl)-benzyl]-([2S,3S]-2-phenyl-piperidin-3-yl)-amine (US 5,703,240, U.S. 5,843,966)

T-type IC50	L-type IC50
~200 nM	> ~2 uM

Compound D: (1S,2S)-2-(2-((3-(2-benzimidazolylpropyl)methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxyacetate (US 4,808,605)

T-type IC50	L-type IC50
~2.7 uM	~19 uM

With respect to the State of the Art, Applicants direct the Examiner's attention to the enclosed reference (Lory, et al., Expert Opin. Ther. Targets (2007), 11(5) 717-722). As noted on page 718 therein, although mibefradil was initially believed to be a selective T-type calcium channel antagonist, it is now admitted that mibefradil potently inhibits many other ion channels, including L-type calcium channels as well as store-operated calcium channels. As further noted in the reference that is cited in Footnote #18 of Lory, et al. (Leuranguer, V., et al., Journal of Cardiovascular Pharmacology, 37 (6), 649-661, Published: June 2001, "Inhibition of T-type and L-type calcium channels by mibefradil: Physiologic and pharmacologic bases of cardiovascular effects.") such teachings were known in the art by at least June 2001.

With respect to the Nature and predictability of the invention, Applicants note that the specification demonstrates that selective T-type calcium channel antagonists possess physiological activity and are useful in accordance with the claimed invention for reducing the number of awakenings during sleep in a mammalian patient.

With respect to the Quantity of Experimentation necessary, Applicants note that methods to select the subject compound and formulate and administer it to reduce the number of awakenings during sleep in a mammalian patient are fully described in the specification.

Accordingly, the rejection of Claims 351-60 under 35 U.S.C. §112, first paragraph, for lack of enablement is untenable and should be withdrawn.

II. Rejection of Claims 51-60 under 35 U.S.C. § 103(a)

Claims 51-60 stand rejected under 35 U.S.C. § 103(a) as being obvious over Branca (U.S. Patent No. 4,808,605) in view of Snutch et al. (WO 01/02561)

The Applicants respectfully traverse this rejection and provide the following comments. The Applicants respectfully assert that Branca in view of Snutch et al. does not disclose or suggest the claimed invention. Nor would Branca in view of Snutch et al. have motivated or enabled one skilled in the art to employ the subject compounds in accordance with the claimed invention. Moreover, in view of the state of the art, one skilled in the art would have been discouraged from the compounds of the claimed invention.

Branca discloses that the compound D (1S,2S)-2-(2-((3-(2-benzimidazolylpropyl)-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxyacetate is a calcium antagonist that may be administered to a patient to treat certain cardiovascular conditions (col. 3, lines 26-30). As the Examiner notes, Branca does not teach that the compound is a T-type calcium channel antagonist. As the Examiner further notes, Branca does not teach that the compound can be used to reduce the number of awakenings during sleep.

Snutch et al. disclose sequences that encode T-type calcium channel subunits α1G subtype, α1H subtype and α1I subtype. Snutch et al. disclose that these subunits can be used to prepare functional calcium channels incorporating these subunits which can be used to evaluate the effects of pharmaceuticals for treating conditions where undesirable T-type calcium channel activity is present, including epilepsy, sleep disorders, mood disorders, cardiac hypertrophy and arrythmia and hypertension.

As the Examiner noted, Snutch et al. do not disclose the specific nature of the sleep disorder (e.g. insomnia, narcolepsy, etc.). Snutch et al. do not indicate whether a T-type calcium channel antagonist would be useful in a sleep disorder where enhancement of sleep is desired (e.g. insomnia) or a sleep disorder where wakefulness is desired (e.g. narcolepsy). In addition, Snutch et al. do not indicate which of the T-type calcium channel subunits α1G subtype, α1H subtype and α1I subtype would be an appropriate target for enhancement of sleep, let alone treating a sleep disorder, in general.

In contrast, the present claims are directed to a method for reducing the number of awakenings during sleep in a mammalian patient by using a T-type calcium channel antagonists that have a specified selectivity and potency, i.e. selectivity for the T-type calcium channel relative to the L-type calcium channel of at least 100 fold, selectivity for the α1I subtype T-type calcium channel relative to the α1G subtype T-type calcium channel of at least 10 fold, selectivity for the α1I subtype T-type calcium channel relative to the α1H subtype T-type calcium channel of at least 10 fold, and potency of an IC_{50} for binding to the T-type calcium channel of 500 nM or less.

Applicants respectively submit that there would have been no motivation nor guidance in Branca in view of Snutch et al. for one of ordinary skill in the art to have attempted to reduce the number of awakenings during sleep in a mammalian patient by using a T-type calcium channel antagonists that have a specified selectivity and potency in accordance with the claimed invention.

Although the compound of Branca was known in the art, there would have been no motivation or suggestion for one of ordinary skill in the art to have determined whether or not it was a selective T-type calcium channel antagonist, nor have believed that the compound could be used to reduce the number of awakenings during sleep.

In fact, Branca in view of Snutch et al. teach away from the present invention by suggesting that general activity as a "calcium antagonist" or that activity at all of the subunits α1G subtype, α1H subtype and α1I subtype would have been desired.

Accordingly, Applicants respectfully submit that the rejection of Claims 51-60 under 35 U.S.C. § 103(a) as being obvious over Branca in view of Snutch et al. is untenable and should be withdrawn.

Applicants respectfully contend that the application is allowable and a favorable response from the Examiner is earnestly solicited.

Respectfully submitted,

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Inhibition of T-Type and L-Type Calcium Channels by Mibefradil: Physiologic and Pharmacologic Bases of Cardiovascular Effects

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Summary: Ca^{2+} channel antagonists of the dihydropyridine, benzothiazepine, and phenylalkylamine classes have selective effects on L-type versus T-type Ca^{2+} channels. In contrast, mibefradil was reported to be more selective for T-type channels. We used the whole-cell patch-clamp technique to investigate the effects of mibefradil on T-type and L-type Ca^{2+} currents (I_{CaT} and I_{CaL}) recorded at physiologic extracellular Ca^{2+} in different cardiac cell types. At a stimulation rate of 0.1 Hz, mibefradil blocked I_{CaT} evoked from negative holding potentials (HPs) (−100 mV to −80 mV) with an IC_{50} of 0.1 μM in rat atrial cells. This concentration had no effect on I_{CaL} in rat ventricular cells (IC_{50}: ~3 μM). However, block of I_{CaL} was enhanced when the HP was depolarized to −50 mV (IC_{50}: ~0.1 μM). Besides a resting block, mibefradil displayed voltage- and use-dependent effects on both I_{CaT} and I_{CaL}. In addition, inhibition was enhanced by increasing the duration of the step-depolarizations. Similar effects were observed in human atrial and rabbit sinoatrial cells. In conclusion, mibefradil combines the voltage- and use-dependent effects of dihydropyridines and benzothiazepines on I_{CaL}. Inhibition of I_{CaL}, which has probably been underestimated before, may contribute to most of the cardiovascular effects of mibefradil. **Key Words:** Mibefradil—Heart—Calcium currents—Inotropism—Bradycardia.

Voltage-gated Ca^{2+} channels are widely expressed throughout the myocardium. The L-type Ca^{2+} channels play a central role in regulation of the action potential (AP) duration, excitation-contraction coupling, and modulation of pacemaker activity (1–3). During the fast upstroke of the AP, they are gated into an open state by membrane depolarization and, thereby, Ca^{2+} influx into the cell. This Ca^{2+} influx has an electrogenic role, contributing to the AP plateau, and constitutes a chemical signal to trigger release of intracellular Ca^{2+} stored in the sarcoplasmic reticulum. The subsequent elevation of free cytosolic Ca^{2+} ions ultimately results in activation of contractile proteins. The role of T-type Ca^{2+} channels is more obscure. They may be involved in cell growth during postnatal development, the regulation of pacemaker activity, or the development of hypertrophy (1–4).

Because of their central role in excitation-contraction coupling, L-type Ca^{2+} channels are a key target to regulate inotropy in the treatment of cardiovascular diseases. The so-called Ca^{2+} channel antagonists have been widely

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used for many years in the treatment of hypertension and angina pectoris (5,6). These Ca^{2+} channel blockers form a heterogeneous class of drugs that fall into three distinct families, each with different binding sites on the channel protein: the dihydropyridines (DHPs; nifedipine, nitrendipine), the phenylakylamines (PAAs; verapamil, D600), and the benzothiazepines (BTZs; diltiazem) (7,8). Recently, mibefradil (Ro 40–5967), a tetralol derivative belonging to a structurally and pharmacologically different class, was introduced for clinical use based on its distinctive pharmacologic and cardiovascular profiles (9). Mibefradil is unique because, compared with DHPs, PAAs, and BTZs, this compound preferentially blocks T-type Ca^{2+} channels (10–12). Although no longer commercially available, the main clinical indications for this drug were hypertension, angina pectoris, and, possibly, treatment of heart failure (13–16). Mibefradil has also anti-ischemic effects (17,18) and reduces infarct size (19). In contrast to traditional Ca^{2+} antagonists, mibefradil has been shown to decrease heart rate without negative inotropism, and its action is not associated with reflex activation of neurohormonal and sympathetic systems (20–22). Although this drug was withdrawn from the market because of major metabolic drug interactions independent of Ca^{2+} channel blockade (23), its beneficial therapeutic effects and, more generally, the concept of T-type Ca^{2+} channel antagonism in cardiovascular diseases are of major interest for the development of new molecules. We used the whole-cell patch-clamp technique to evaluate the effects of mibefradil on T-type and L-type Ca^{2+} channels in different kinds of cardiomyocytes, including rat cardiomyocytes, rabbit sinoatrial cells, and human atrial cells.

METHODS

Rat cardiomyocytes

This investigation conforms with the guide for the care and use of laboratory animals published by the European Community (96/609/EEC). Atrial and ventricular myocytes were isolated enzymatically from Wistar–Kyoto rat hearts. Animals aged 8 days and 6–8 weeks of either sex were anesthetized with 30 mg/kg of pentobarbital sodium injected intraperitoneally. The heart was quickly removed and rinsed in a warm Tyrode solution containing (in m*M*): 112 NaCl, 6 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 4 $NaHCO_3$, 1.5 KH_2PO_4, 25 HEPES, 10 pyruvic acid, 5.85 glucose, 17.7 mg/l phenol red, 60 mg/l penicillin G, and 100 mg/l streptomycin (pH 7.4 adjusted with NaOH). The heart was then perfused retrogradely through the aorta (at 37°C) with a Ca^{2+}-free Tyrode solution for 8 min and again with the same solution supplemented with 10 μM Ca^{2+} and 0.7 mg/ml collagenase (type 2; Worthington Biochemical, Lakewood, NJ, U.S.A.) for 15–30 min. After this step, myocytes were dissociated mechanically with a smooth Pasteur pipette. Then cells were stored for 6–8 h in a high potassium medium containing (in m*M*): 100 K^+-glutamate, 10 K^+-aspartate, 25 KCl, 10 KH_2PO_4, 2 $MgSO_4$, 20 taurine, 5 creatine, 0.5 ethyleneglycotetraacetic acid, 20 glucose, 5 HEPES, and 0.1% bovine serum albumin (BSA) (pH 7.4 adjusted with KOH) at 4°C.

Human atrial cells

Small fragments of the right atrial appendage (0.5–1 cm^2) were obtained during open heart surgery (before cardiopulmonary bypass) in accordance with the institutional guidelines for human subject research. Clinical diagnosis was coronary artery disease (four patients). Patients had previously received medication including β-adrenergic blockers or angiotensin-converting enzyme inhibitor. The solutions used for transportation and the enzymatic isolation procedure (0.5 mg/ml protease [type 14, Sigma, St. Louis, MO, U.S.A.], 0.6 mg/ml collagenase [type H], *Clostridium histolyticum*, and 0.2 mg/ml elastase, [Boehringer Mannheim, Mannheim, Germany]) have been detailed previously (24). Briefly, the tyrode composition for dissociation and transportation was (in m*M*): 136 NaCl, 10.8 KCl, 1.1 $MgCl_2$, 22 dextrose, 25 HEPES, 10 glutamate, 0.01 $CaCl_2$, 60 μg/ml penicillin, 100 μg/ml streptomycin, and 0.002% phenol red indicator (pH adjusted to 7.4 with NaOH). After the enzymatic procedure, the dissociation was achieved by mechanical agitation and cells were stored in the same solution as used for rat ventricular cells. Myocytes were dispersed by mechanical agitation in the recording chamber just before electrophysiologic experiments. Only rod-shaped, well-relaxed, striated myocytes were used for recording.

Rabbit sinoatrial cells

Spontaneously beating sinoatrial cells were isolated from young albino rabbits (0.8–1 kg) as described before (25). Briefly, beating hearts were removed under pentobarbitone (360 mg/kg, Sanofi, Santé Nutrition Animale, Libourne, France) and ketamine (50 mg/kg, Sanofi) anesthesia. The sinoatrial region was excised in a normal Tyrode solution containing (in m*M*): 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES-NaOH, and 5.5 D-glucose (pH at 7.4). Tissues were enzymatically digested in a low-Ca^{2+}, low-Mg^{2+} solution containing (in m*M*): 140 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 0.2 $CaCl_2$, 1.2 KH_2PO_4, 50 taurine, 5.5 D-glucose, and 5 HEPES-NaOH, (pH at 6.9). Collagenase type II (224 U/ml, Worthington), elastase (1.9 U/ml, Worthington), and BSA 1 mg/ml were added. Digestion step was carried out for approximately 15 min

under gentle mechanical agitation at 36°C. Tissue was then washed out and transferred into a modified Kraft-brüe (KB) solution containing (in m*M*): 70 L-glutamic acid, 20 KCl, 80 KOH, 10 D-β-OH-butyric acid, 10 KH_2PO_4, 10 taurine, 10 HEPES-KOH, and 1 mg/ml BSA (pH at 7.4). Single sinoatrial myocytes were manually dissociated in KB solution by employing a flame-forged Pasteur pipette. Finally, cell automaticity was recovered by gradually increasing extracellular Ca^{2+} up to 1.3 m*M*. The final storage solution contained (in m*M*): 100 NaCl, 50 KCl, 1.3 $CaCl_2$, 0.7 $MgCl_2$,1 mg/ml BSA, and 50 μg/ml gentamycin (pH at 7.4). Cells were then stored at 4°C until use.

Electrophysiologic recordings

The waveforms of I_{CaL} were measured 2–10 h after cell dispersion using the whole-cell patch-clamp technique by means of an Axopatch 200A patch-clamp amplifier (Axon, Foster City, CA, U.S.A.). Experiments were performed at room temperature (22°C–24°C) to slow current activation and minimize current amplitude. Recording conditions were optimized to eliminate contaminating voltage-gated inward Na^+ and outward K^+ currents. The intracellular recording solution contained (in m*M*): 130 CsCl, 10 ethyleneglycotetraacetic acid, 25 HEPES, 3 ATP(Mg), 0.4 GTP(Na), pH 7.2 adjusted with CsOH. Cells were recorded in a bath solution containing (in m*M*): 2 $CaCl_2$, 5 4-aminopyridine, 136 TEA-Cl, 1.1 $MgCl_2$, 25 HEPES, and 22 glucose (pH 7.4 adjusted with TEAOH; 290–310 mOsm). The patch pipettes had a resistance of 2–3 MΩ; when filled with the recording solution. After membrane disruption, series resistances (estimated from the decay of the uncompensated capacitive transients) were approximately 1.5–2 times the pipette resistance (<6 MΩ). Residual series resistance after electronic compensation (80%) were ≤1.5 MΩ. Experimental parameters, such as HPs, test potentials, and sampling intervals were controlled with an IBM PC connected to a Digidata 1200 interface (Axon). Data acquisition and analyses were performed using the pClamp software (version 6.03, Axon). Signals were filtered at 3–5 kHz before digitization and storage. I_{CaL} was recorded generally at a test pulse ranging between −10 mV and +10 mV delivered from various HPs as quoted in figure legends. I_{CaT} was recorded at a test pulse of −30 mV delivered from a HP of −100 mV. I_{CaL} and I_{CaT} were measured as the difference between the maximal inward current amplitude and the zero current level. All averaged or normalized data are presented as mean ± SD. To determine steady-state inactivation curves, peak currents evoked at −30 mV (for I_{CaT}) or 0 mV (for I_{CaL}) were measured after 5-s conditioning HPs and were normalized to the current elicited at −100 mV (for I_{CaT}) or −80 mV (for I_{CaL}). The fraction of channels not inactivated (h) was plotted versus voltage (availability curve). The fitted curves were described by the equation

$$h = \frac{1}{[1 + \exp(V - V_{1/2})/k]},$$

where $V_{1/2}$ indicates voltage for half-inactivation and k indicates the slope.

Statistical analysis

All averaged or normalized data are presented as mean ± SD. The significance between groups of data was assessed using Student's t test (for paired or unpaired samples as appropriate). Results were considered not significant (NS) with $p > 0.05$ and significant (*) with $0.01 < p < 0.05$

Solutions

Mibefradil was prepared extemporaneously as 10 m*M* stock solutions in H_2O and subsequently diluted at the desired working concentrations in test solutions. Control and test solutions were applied by using a multiple-capillary gravity-driven perfusion system (inner diameter, 200 μm; flow rate, 0.5 ml/min) placed in the vicinity of the cell (< 0.5 mm). Each capillary was fed by a reservoir 50 cm above the bath. Rapid (at most seconds) and complete solution changes could be made by switching from the opening from one capillary to the next.

Numerical simulation of action potential in sinoatrial cells

For numerical modeling of the sinoatrial automaticity, we adapted the original equations for I_{Ca} used by Noble et al. (26) in their single-cell model of sinoatrial automaticity. Calculations were carried out using the 4.8 version of the Oxsoft Heart program (27). To account for all voltage-, time-, and Ca^{2+}-dependent properties of Ca^{2+} channels, we adapted a Markovian model for I_{CaL} (28).

RESULTS

Effect of mibefradil on I_{CaT}

We studied the effect of mibefradil on I_{CaT} in cells freshly isolated from the atria of young rats (8 days after birth). These myocytes express robust I_{CaT} (29). I_{CaT} could be recorded in complete isolation at depolarizations (−30 mV) that activated maximal current but were too weak to activate significant I_{CaL}. This I_{CaT} was highly sensitive to mibefradil. The concentration-response curve (Fig. 1A) shows an IC_{50} of 0.1 μ*M*. In-

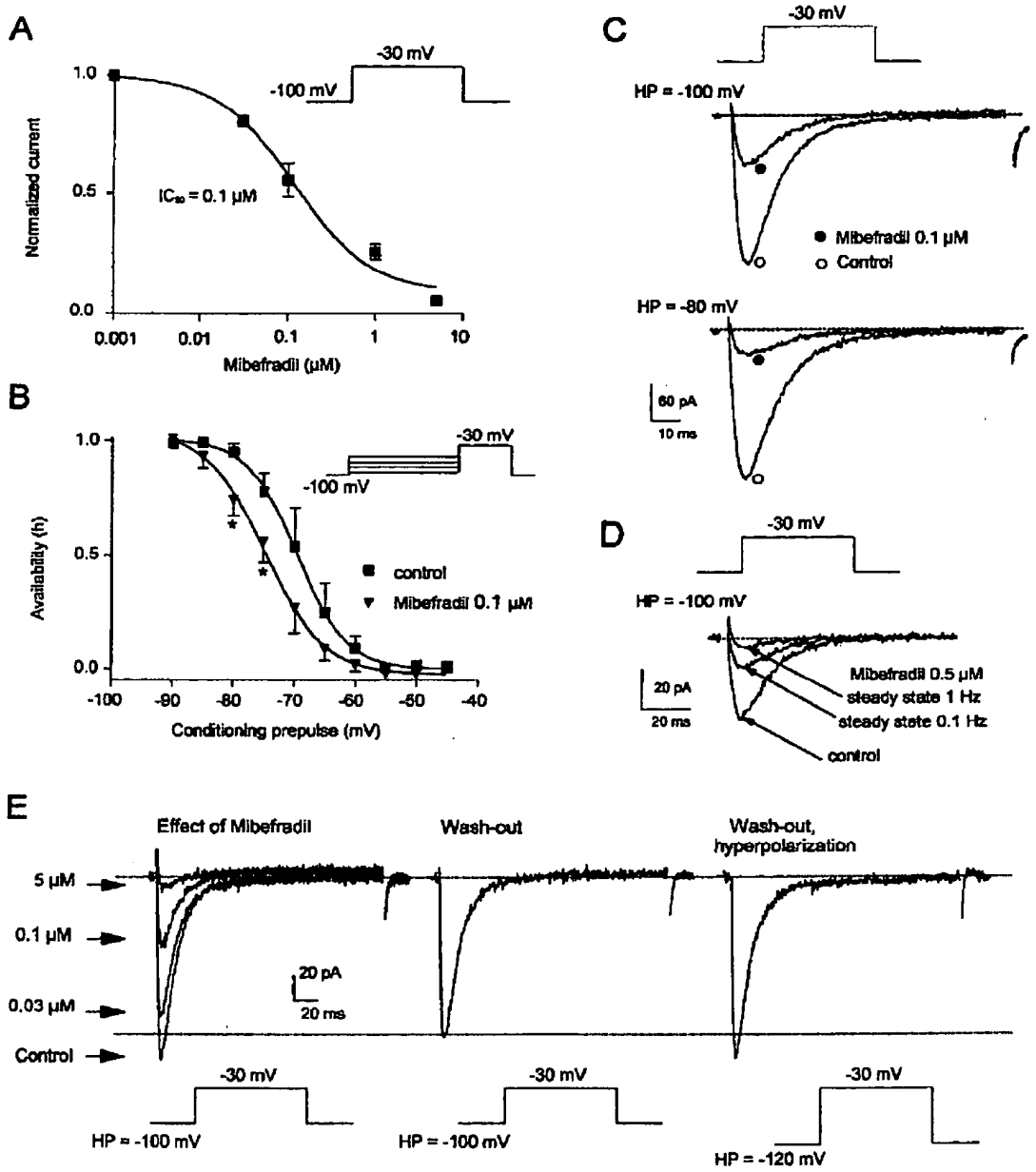


FIG. 1. Voltage- and use-dependent block of I_{CaT} by mibefradil in 8-day-old rat atrial cells. **A.** Cumulative dose-response curve for I_{CaT} measured at −30 mV from holding potentials (HPs) −100 mV (mean ± SD; 11 cells). The IC_{50} was 0.1 µ*M*. **B.** Voltage-dependent inactivation curves of I_{CaT} (see Methods) in absence and presence of 0.1 µ*M* mibefradil (mean ± SD; n = 3). *$p < 0.05$. **C.** Example of I_{CaT} evoked at −30 mV from HPs −100 mV and −80 mV, respectively, in absence and presence of 0.1 µ*M* mibefradil. **D.** Example of the effects of an increase in the rate of stimulation on mibefradil inhibition of I_{CaT}. I_{CaT} was recorded at 0.1 Hz in presence of 0.5 µ*M* mibefradil until the equilibrium was reached; then the rate of stimulation was increased to 1Hz. **E.** Cumulative effects of increasing concentrations of mibefradil until complete block of I_{CaL} and wash-out. Incomplete wash-out was obtained after 3 min, when I_{CaL} was evoked from an HP of −80 mV. However, full recovery occurred immediately (at the first stimulation) when the HP was hyperpolarized to −120 mV.

terestingly, mibefradil induced a shift (5.2 ± 1.1 mV) in the steady-state inactivation curve toward negative potentials (Fig. 1B). This effect is consistent with a higher affinity of the drug for the inactivated state of the T-type Ca^{2+} channel. This shift also shows that the effect of mibefradil on I_{CaT} is voltage-dependent and, therefore, conditioned by the resting membrane potential from which the currents are evoked (Fig. 1C). The block induced at an HP of −100 mV by a given concentration of mibefradil was enhanced at an HP of −80 mV. Mibefradil inhibition of I_{CaT} was also accentuated by an increase in the frequency of repetitive membrane step depolarizations (use-dependent or frequency-dependent block). Figure 1D illustrates, for example, the typical additional blocking effect of 0.5 μM mibefradil on I_{CaT} when the rate of stimulation was increased from 0.1 Hz to 1 Hz. The inhibition was only partial at 0.1 Hz and nearly complete at 1 Hz. This enhancement was reversible when the diastolic interval was increased (data not shown), suggesting that low frequencies also favor unbinding of the drug. Recovery is illustrated in Figure 1E. In this experiment, I_{CaT} was submitted to cumulative concentrations of mibefradil until nearly complete blockade, which occurred at 5 μM. Recovery was observed after 2-min wash-out of the drug but was slow and only partial, probably because of accumulation of the drug within the cell membrane. However, complete recovery occurred within seconds when the HP was hyperpolarized to −120 mV, suggesting that hyperpolarization favored immediate unbinding of some drug still present on its receptor.

Effect of mibefradil on I_{CaL}

We next studied the influence of the HP on the inhibitory effect of mibefradil on I_{CaL}. Experiments were performed in adult rat ventricular myocytes because they express robust I_{CaL} routinely and I_{CaT} is completely absent (1). We found that I_{CaL} is also sensitive to mibefradil, but, when evoked from negative HPs, inhibition was observed at concentrations higher than those sufficient to block I_{CaT} (Fig. 2A). The IC_{50} was 3.4 μM when the currents were evoked from a HP of −80 mV. However, inhibition of I_{CaL} by mibefradil was markedly enhanced by depolarizing the HP. For example, inhibition was more potent at an HP of −50 mV than at an HP of −80 mV (Fig. 2A). Figure 2B shows the corresponding averaged concentration-response curves. Interestingly, half-inhibition of I_{CaL} evoked from an HP of −50 mV occurred for a concentration that had only little effect when the current was evoked from an HP of −80 mV. The IC_{50} values were 3.4 μM and 0.1 μM for an HP of −80 mV and −50 mV, respectively. As expected, mibefradil induced a leftward shift in the steady-state inactivation curve of I_{CaL} (Fig. 2C). The $V_{1/2}$ shifted from −32.9 ± 1.1 mV in control conditions to −39.4 ± 1.2 mV ($p < 0.001$; $n = 7$) in the presence of mibefradil. This observation indicated that drug affinity is higher for the inactivated state than for the resting state of the L-type Ca^{2+} channel.

We assessed whether there was a use-dependent block of I_{CaL}. Figure 3A shows that application of mibefradil at a concentration of 5 μM for 2 min and in absence of any stimulation produced only partial inhibition of I_{CaL}. However, when a train of depolarizations at 1 Hz was applied, an additional block occurred that led to an almost complete inhibition of I_{CaL}. This new steady state was achieved within a few stimulations (i.e., within a few seconds), indicating that the cumulative effect of stimulation is in fact a genuine effect of the drug and does not reflect rundown of the current. The lack of effect of increased frequency per se on current peak amplitude and the different kinetics of inhibition are illustrated in Figure 3B. Moreover, the two types of inhibition were easily removed on wash-out of the drug, and after hyperpolarization of the HP to −120 mV for at least 1 min (data not shown). We found that block of I_{CaL} at a given rate was also dependent on the duration of the depolarizing pulse. An increase in the duration of the test pulse indeed increased the fraction of inactivated channels at the end of each depolarizing test pulse. Figure 3C shows that inhibition was enhanced by application of longer test depolarizations, which is consistent with higher affinity of mibefradil for the inactivated state of the L-type Ca^{2+} channel.

Inhibition of I_{CaL} by mibefradil in human atrial cells

We next assessed whether our data in rat cardiac myocytes were transposable to human cardiomyocytes. This was tested only on I_{CaL} because no I_{CaT} was recorded in this particular study. The lack of I_{CaT} has been a general finding over a period of 10 years in all laboratories investigating I_{CaT} in human cardiomyocytes (1). Nevertheless, we have previously reported the presence of a low-voltage-activated tetrodotoxin-sensitive Ca^{2+} current ($I_{Ca,TTX}$) in some human atrial cells. In the current study, there was no contamination of I_{CaL} by I_{CaTTX} for two reasons: (a) at the test depolarization used here (−10 mV), I_{CaTTX} has only weak peak amplitude; and (b) we used an HP of −80 mV, which inactivates more than 90% of this current (30). Otherwise, I_{CaTTX} can be easily detected owing to its very fast kinetics (much faster than that of I_{CaL} or even I_{CaT}). When I_{CaL} was evoked from a negative HP (−80 mV) and at low rate of stimulation

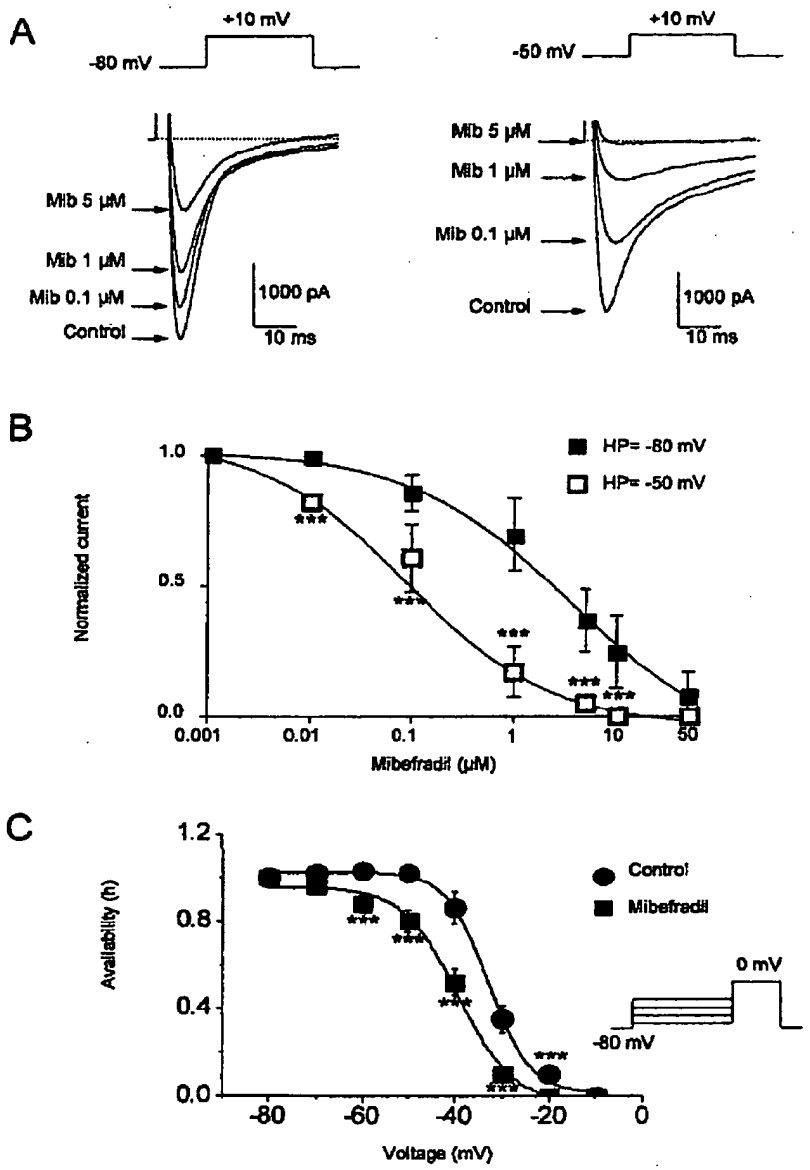


FIG. 2. Modulation of mibefradil effect on I_{CaL} by membrane potential in adult rat ventricular myocytes. A. Typical effect of increasing concentrations of mibefradil (Mib) on I_{CaL} recorded from two different holding potentials (HPs) at −80 mV (left panel) and −50 mV (right panel). The decay kinetics of I_{CaL} were slower at an HP of −50 mV, a phenomenon due to some type of facilitation of Ca^{2+} channel activity by moderate depolarization of the diastolic membrane potential in normal cells (48). B. Cumulative dose-response curves of I_{CaL} obtained at two different HPs (mean ± SD; n = 7 and n = 8, respectively). ***p < 0.001. C. Steady-state voltage-dependent inactivation curves of I_{CaL} (see Methods) in absence and presence of 1 µM mibefradil (mean ± SD; 7 cells) ***p < 0.001.

(0.1 Hz), mibefradil had no significant effect at 0.1 μM in all of five cells tested (Fig. 4Aa). As observed in rat cardiomyocytes, inhibition of I_{CaL} was enhanced at less negative HPs (Fig. 4Ba) and at the higher rate of stimulation (Fig. 4Ab). On average, 1 μM mibefradil blocked 37 ± 10% (n = 3) of I_{CaL} peak amplitude when I_{CaL} was evoked at a stimulation frequency of 0.1 Hz from an HP of −80 mV. This percentage was much increased when the stimulation frequency was accelerated to 1 Hz (70 ± 8%) or when the HP was depolarized to −60 mV (62 ± 8%; n = 3). Strikingly, complete block (96 ± 3%) occurred when sustained depolarization to −60 mV (HP) and stimulation at 1 Hz were combined (see Fig. 4Bb).

Inhibition of I_{CaL} by mibefradil in rabbit pacemaker sinoatrial cells

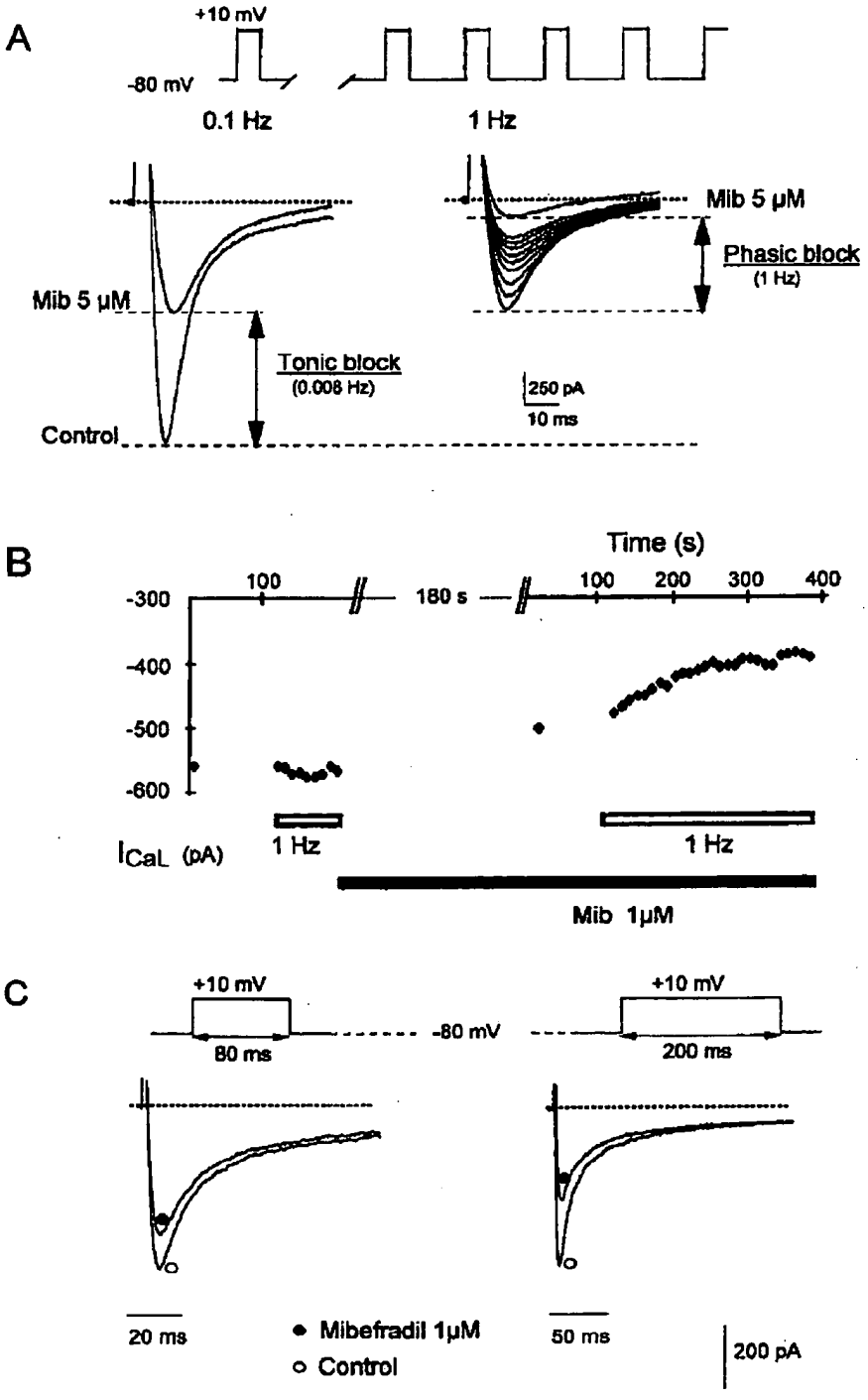
Because mibefradil induces a slight decrease in heart rate, we were interested in studying the potential involvement of I_{CaL} inhibition in bradycardia. Experiments were performed in spontaneously beating spindle-shaped rabbit sinoatrial cells. Step depolarizations delivered from an HP of −100 mV could lead to the activation of two types of currents corresponding to I_{CaT} and I_{CaL}, as described before (1–4,28). However, in contrast to I_{CaL}, which was detected in nearly all cells (24 of 25 cells), I_{CaT} was barely observed (only in four cells). In addition, its amplitude was very small (<20 pA; i.e., less than 20% of that of I_{CaL}), suggesting that most cells can beat spontaneously without the help of I_{CaT}. Because of its rare occurrence and weak peak amplitude, the effect of mibefradil was not investigated on I_{CaT} in sinoatrial cells. We found that I_{CaL} could also be inhibited by mibefradil. No inhibition occurred at 0.1 μM when I_{CaL} was evoked at a frequency of stimulation of 0.1 Hz and from an HP of −100 mV (Fig. 5Aa). In these conditions, however, 1 μM mibefradil (a concentration corresponding to the IC_{50} in other cardiac cell types) inhibited 50% of I_{CaL} in rabbit sinoatrial cells. The block was both voltage- and use-dependent. The inhibitory effect of the drug was enhanced by applying repetitive stimulation at higher rates (Fig. 5Ab). On average, mibefradil inhibited 45 ± 7% and 81 ± 5% of I_{CaL} (evoked from an HP of −80 mV) at 0.1 μM and 1 μM (n = 5), respectively. In similar conditions, but with a less negative HP (−60 mV), 1 μM mibefradil inhibited 100% of I_{CaL} at 1 Hz.

DISCUSSION

Mibefradil acts as an antihypertensive drug and promotes bradycardia without reducing the contractile force of the myocardium. In this study, we examined the effects of mibefradil on T- and L-type Ca^{2+} channels in the context of cardiac pathophysiology. The main results are (a) mibefradil is not highly selective for I_{CaT} because high-affinity block can also occur on I_{CaL} depending on the experimental conditions; (b) inhibition of I_{CaT} and I_{CaL} is markedly voltage- and use-dependent; and (c) mibefradil blocks I_{CaL} similarly in all types of cardiomyocytes tested, including rat ventricular, human atrial, and rabbit sinoatrial cells. These properties may account for most of the cardiovascular profile of mibefradil.

We show here that mibefradil blocks I_{CaT} more effectively than I_{CaL} in rat cardiomyocytes. Inhibition occurs at submicromolar concentrations (IC_{50}: 100 nM), as also described in vascular smooth muscle cells (10), human medullar thyroid carcinoma (11), and guinea-pig cardiomyocytes (12,15,31), suggesting that T-type Ca^{2+} channels have high-affinity binding sites. However, mibefradil is not a selective T-type channel blocker. We demonstrate here that I_{CaL} can also be inhibited at submicromolar concentrations. There is a 30-fold change in the IC_{50} (3.4 vs. 0.1 μM) when the HP is depolarized from −80 to −50 mV. This is better explained by the increased affinity of the drug for its binding receptor as a consequence of voltage-dependent conformational changes in the L-type Ca^{2+} channel protein. Thus we conclude that high-affinity binding of mibefradil is not a hallmark of T-type Ca^{2+} channels. Consistent with this idea, various classes of neuronal HVA Ca^{2+} channels can also be blocked at low concentrations (31–34).

Pharmacokinetics, channel specificity, distinct binding sites, and voltage- and use-dependent effects lead to major differences in the profile of DHPs, PAAs, and BTZs (35). They are currently distinguished by their binding sites and their mode of action, which have interesting quantitative differences in the extent to which they display use-dependence (35–37). For example, the effects of verapamil, D600, and diltiazem, which are tertiary amines, are much more use-dependent, in part because of blockade of open channels, than the effects of most DHPs that are uncharged at physiologic pH (36,37). In contrast with the effects of PAAs and BTZs, which resemble the block of Na^+ channels by tertiary amine local anesthetics, and in accordance with the modulated receptor scheme (38,39), voltage-dependent block of DHPs occurs at submicromolar concentration in the absence of repetitive depolarizations (36,37,40). We demonstrate here that, in addition to acting through a verapamil-type binding site (8,21), mibefradil has interesting mixed use- and voltage-dependent effects on cardiac I_{CaL}. Submicromolar concentrations of mibefradil are clearly ineffective when Ca^{2+} channels are gated from hyperpolarized HPs (i.e., on closed channels), particularly at low



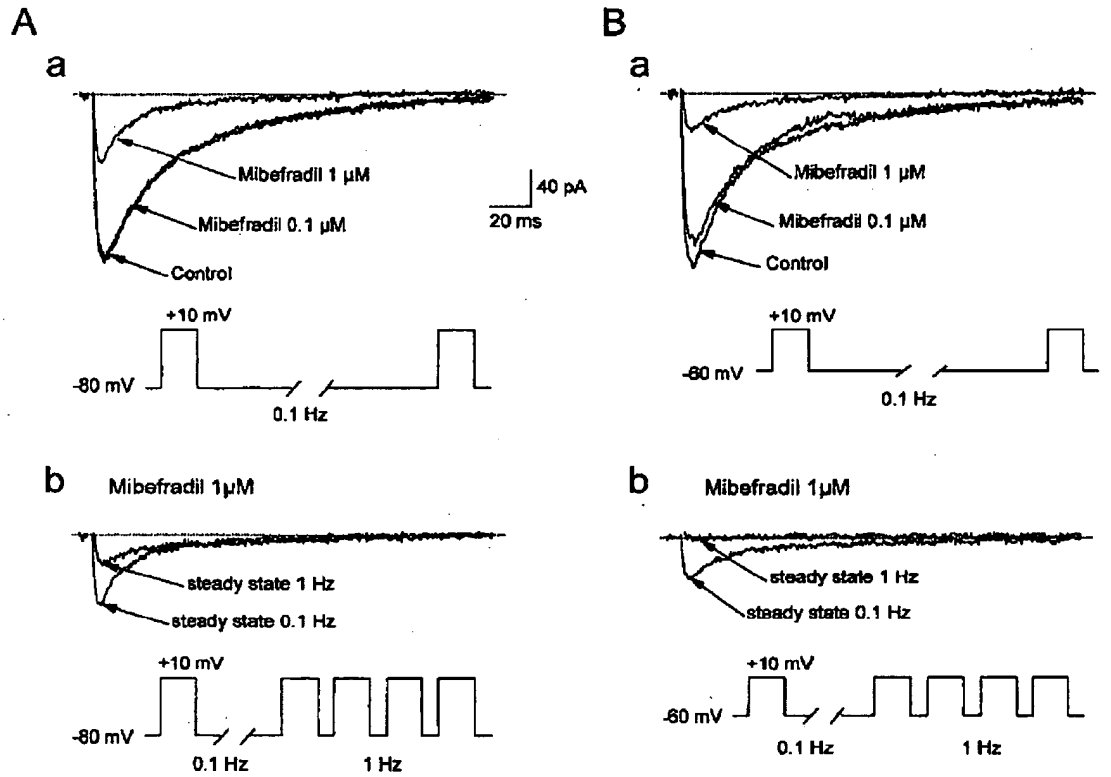


FIG. 4. Mibefradil inhibition on I_{CaL} in atrial human myocytes. **A.** Representative traces of I_{CaL} evoked at −10 mV from a holding potential (HP) of −80 mV. **a.** Application of 0.1 μ*M* mibefradil had no effect at a depolarization rate of 0.1 Hz. Substantial inhibition of I_{CaL} (>50%) occurred at 1 μ*M*. **b.** Steady-state block at 0.1 μ*M* was enhanced upon increasing the depolarization rate to 1 Hz. **B.** Effect of changing the HP to −60 mV. Mibefradil inhibition was enhanced (as compared with that observed at an HP of −80 mV) at 0.1 Hz (**a**) and 1 Hz (**b**). All data were obtained in the same cell. The blocking effect observed in **A** was removed upon wash-out of the drug, and hyperpolarization of the HP to −120 mV for 1 min (data not shown), as indicated by comparing the amplitude of the control currents in **A** and **B**. Control currents were not affected by the increase in the rate of stimulation (neither peak amplitude nor the decay kinetics). Similar effects were observed consistently in four other cells.

rates of stimulation. Indeed, as reported for BTZs and PAAs (36), we found that mibefradil blockade is use-dependent and requires repetitive openings. Nevertheless, an increase in the length of step-depolarizations, which promotes time-dependent inactivation, and, more importantly, steady-depolarizations dramatically potentiate the blocking effect (as reported for DHPs). Thus this voltage-dependent block strongly depends on cell resting

FIG. 3. Block of I_{CaL} by mibefradil depends on frequency and duration of test depolarization. **A.** Tonic block: steady-state inhibitory effect of mibefradil (Mib) applied for 2 min in the absence of stimulation. Phasic block: additional blocking effect of the drug induced by stimulating the cell at 1 Hz, a rate of stimulation that had no effect on control condition. The upper trace was obtained at steady-state after 30 s. Similar effects were observed consistently in five other cells. **B.** Tonic and phasic block of I_{CaL} after exposure to 1 μ*M* mibefradil, in the absence of stimulation for 3 min and, then, during repetitive stimulations at 1 Hz. Note that there is no major change in current peak amplitude at 1 Hz in the absence of drug. **C.** I_{CaL} recorded at +10 mV from a holding potential (HP) of −80 mV, at 0.1 Hz. Inhibition by 1 μ*M* mibefradil was enhanced when pulse duration was increased from 80 ms (21%) to 200 ms (46%). Steady state was achieved after 10 stimulations. No effect was observed in control conditions. Data were obtained in the same cell. The blocking effect observed in the left panel was easily removed upon wash-out of the drug, and hyperpolarization of the HP to −120 mV for 1 min (data not shown), as indicated by the amplitude of the control currents in the right panel. Similar effects were observed consistently in three other cells.

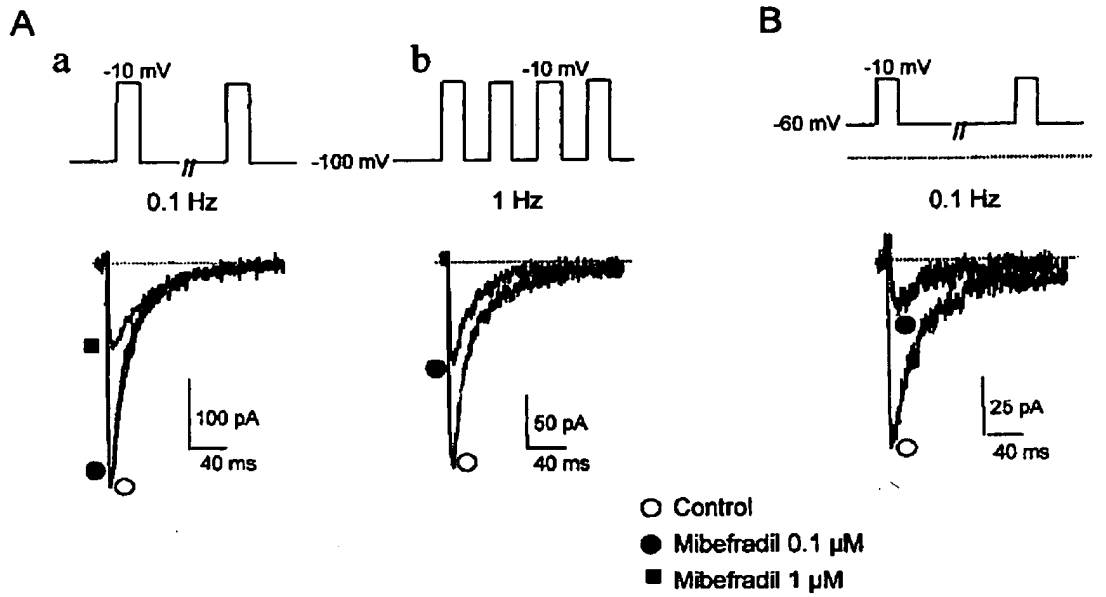


FIG. 5. Block of I_{CaL} by mibefradil in rabbit pacemaker cells. A. I_{CaL} was evoked at −10 mV from a holding potential (HP) of −100 mV (top). a. At 0.1 Hz, 0.1 μM mibefradil had no significant effect, but substantial block (> 50%) occurred at 1 μM. b. However, 0.1 μM mibefradil also blocked I_{CaL} substantially when the depolarization rate was increased to 1 Hz. Data in a and b were collected from the same cell. The blocking effect observed in a was recovered upon wash-out of the drug, and hyperpolarization of the HP to −120 mV for 1 min (data not shown), as indicated by comparing the amplitude of the control currents in a and b. B. Mibefradil induced very substantial block of I_{CaL} at an HP of −60 mV. Effects similar to that shown in A and B were observed consistently in three other cells.

potentials. As a corollary, unblock is favored by hyperpolarization and slower rates of stimulation.

Our data suggest that mibefradil has a substantial effect on the inactivated state of the cardiac L-type Ca^{2+} channel, although it probably also displays open block, in agreement with previous reports on cloned Ca^{2+} channels (41–43). We find that there is a high degree of similarity between the action of mibefradil and the known effects of PAAs and BTZs. It has been proposed that all these drugs have access to their receptor via the open channel conformation (32). Slow recovery from open channel block may indeed account for the use-dependent block (32). However, we present here also strong evidence for a higher affinity of the drug for the inactivated state. Thus there is also some similarity between the effect of mibefradil, which is, for example, more voltage-dependent than that of verapamil (44), and most DHPs. Mibefradil shifts the steady-state inactivation curve of I_{CaL} and inhibition is more pronounced when Ca^{2+} channels are operated from depolarized HPs. The difference (30- to 70-fold) reported before in the apparent affinities of resting and inactivated states of recombinant Ca^{2+} channels (41), which is based on a modulated receptor hypothesis, is quite similar to the 30-fold change in the IC_{50} that we find here for Ca^{2+} channels examined in native cardiac cells. Interestingly, we also show here that mibefradil's effect on T-type Ca^{2+} channels undergoes similar modulation by voltage and frequency of stimulation, which contrasts with results obtained in a human medullar thyroid carcinoma cell line (11).

Our study suggests that the potency of mibefradil inhibition on I_{CaL} is modulated by the electrical profile of the cells. For example, at submicromolar concentrations that are not high enough to depress I_{CaL}, and thereby the AP plateau at basal conditions, mibefradil is likely to become active if there is an increase in the heart rate, an increase in the AP duration, or, a sustained membrane depolarization. This property may be particularly important to protect the heart from catecholaminergic overstimulation that increases the AP duration and accelerates the heart rate. It may contribute to the cardioprotective effect of this drug on ischemic myocardium or in survival of rats with chronic heart failure treated by mibefradil (14,19). In contrast to BTZs and PAAs, which are nonselective (they cause vasodilatation

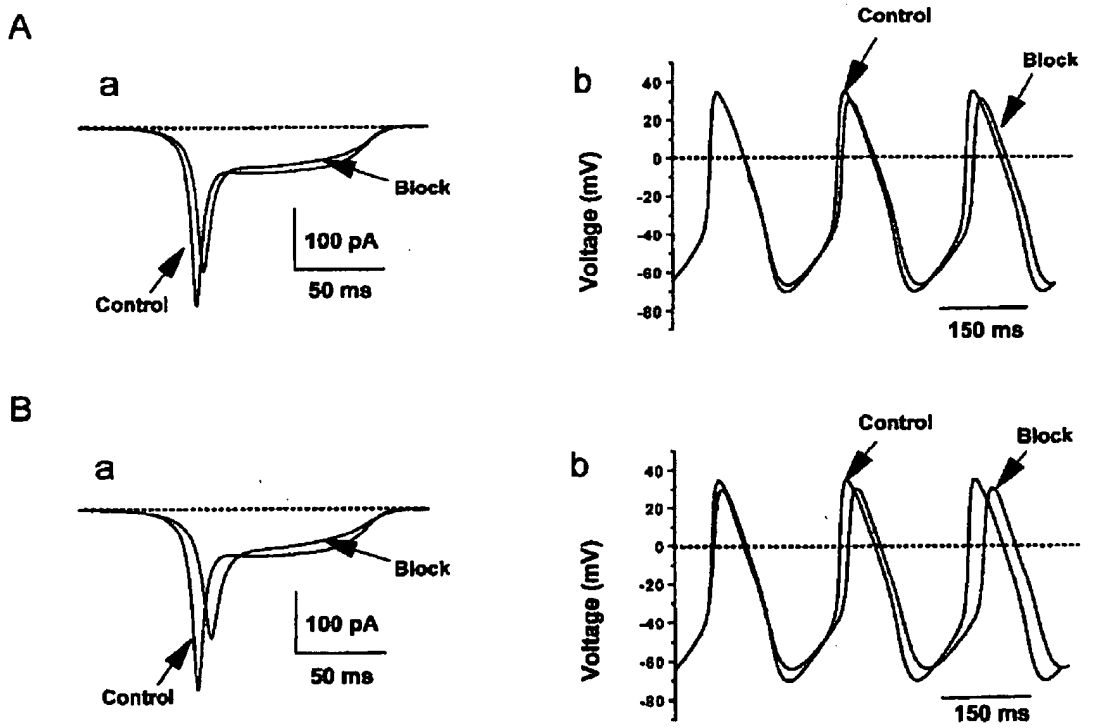


FIG. 6. Numerical simulation of mibefradil effect on sinoatrial automaticity in rabbit. All computations were carried out assuming a membrane diastolic potential of –69 mV and absence of I_{CaT} (see Methods). **Aa.** Computed time course of I_{CaL} during the sinoatrial action potential in control conditions and after assuming a 20% block of I_{CaL} by mibefradil. **Ab.** Slowing of the pacemaker rate corresponding to the block of I_{CaL} shown in **Aa. Ba.** Same computation as in **Aa**, but assuming 40% of block of I_{CaL} by mibefradil, which corresponds theoretically to inhibition by a concentration that has no effect on contractile myocytes (< 0.1 μM). Note the dose-dependent block of I_{CaL}, which slows automaticity and depolarizes the membrane diastolic potential gradually. This latter effect, in turn, further enhances the affinity of mibefradil for I_{CaL}.

and depress cardiac contractility), but similar to second-generation DHPs (35), mibefradil exerts moderate negative inotropic effects at high doses (20,21). It was proposed that the cardiovascular effects of mibefradil reflect a selective inhibition of T-type Ca^{2+} channel (10) for two reasons: (a) mibefradil has no effect on I_{CaL} evoked from hyperpolarized membrane potential (as expected in ventricular myocytes); and (b) mibefradil blocks I_{CaT} efficiently at those potentials. However, there are some difficulties with this theory. First, we (and many other investigators) found no evidence for the presence of I_{CaT} in freshly isolated human cardiac, as well as coronary, myocytes (1,44–46), which suggests that I_{CaT} may not be a key target involved in the therapeutic benefits of the drug at the cardiac level. Second, it seems unlikely that T-type Ca^{2+} channels contribute to significant voltage-dependent Ca^{2+} entry and, thereby, to the maintenance and development of contractile tone in vascular cells. The resting membrane potential of these cells (≥–60 mV) is not negative enough to allow significant activation of I_{CaT}. Therefore, we would not expect any significant effect of mibefradil reflecting T-channel blockade. Enhanced blockade of L-type channels at these potentials may account for the observed cardiovascular effects on depolarized tissues. High-affinity blockade of mibefradil for inactivated Ca^{2+} channels may explain its selectivity for tissues with less negative resting potentials (e.g., vascular and sinoatrial cells vs. ventricular myocytes).

The frequency-dependent effect of mibefradil on I_{CaL} may be sufficient to explain the effect of the drug on heart rhythm without concomitant negative inotropism. Numerical simulations of sinoatrial cell automaticity showed that the increment in the blocking effect of mibefradil on I_{CaL} (20% and 40% inhibition, respectively)

induced a moderate slowing of the frequency of APs (Fig. 6). A shift of the maximal diastolic potential (MDP) to more positive values is a basic prediction of the sinoatrial cellular automaticity model. Indeed, inhibition of I_{CaL} slows the AP upstroke velocity and reduces the AP amplitude. This causes less fractional K^+ current activation during the AP, thus setting the MDP to a more positive value (the net membrane current becomes inward at less negative membrane potential). Accordingly, this condition is experimentally verified, because partial block of I_{CaL} by nifedipine actually reduces the AP amplitude and shifts the MDP to more positive values (47). The voltage dependency of mibefradil block has not been included in this simulation, because at an HP of approximately −70 mV, a value corresponding to the MDP (Fig. 6), the percentage of block of I_{CaL} by 1 μM mibefradil is only 2%. This figure thus highlights the effect of the use-dependent block of I_{CaL} by mibefradil on the cellular automaticity.

In summary, this study shows that mibefradil, which was once thought to be selective for T-type Ca^{2+} currents, has a broader pharmacologic impact for Ca^{2+} channel antagonism. Indeed, mibefradil can also be a potent blocker of L-type Ca^{2+} channels owing to a particular pharmacologic profile that combines the specific effects of two different classes of Ca^{2+} channel antagonists (DHPs and BTZs). As a result, effect of mibefradil on L-type Ca^{2+} channels is enhanced at depolarized diastolic membrane potentials, high heart rates, or AP prolongation, which may account for most of its cardiovascular effects. Although mibefradil has been withdrawn from the market because of highly undesirable drug interactions (23), its intrinsic properties on L-type Ca^{2+} channel should be taken into account for the design and development of new molecules. In addition, the concept of T-type Ca^{2+} channel antagonism to explain the therapeutic benefits of mibefradil may be misleading.

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Abstract: Ca2+ channel antagonists of the dihydropyridine. benzothiazepine, and phenylalkylamine: classes have selective effects on L-type versus T-type Ca2+ channels. In contrast, mibefradil was reported to be more selective for T-type channels. We used the whole-cell patch-clamp technique to investigate the effects of mibefradil on T-type and L-type Ca2+ currents (I-CaT and I-CaL) recorded at physiologic extracellular Ca2+ in different cardiac cell types. At a stimulation rate of 0.1 Hz, mibefradil blocked I-CaT evoked from negative holding potentials (HPs) (-100 mV to -80 mV) with an IC50 of 0.1 muM in rat atrial cells. This concentration had no effect on I-CaL in rat ventricular cells (IC50: similar to3 muM). However, block of I-CaL was enhanced when the HP was depolarized to -50 mV (IC50: similar to0.1 muM). Besides a resting block, mibefradil displayed voltage- and use-dependent effects on both I-CaT and I-CaL. In addition, inhibition was enhanced by increasing the duration of the step-depolarizations. Similar effects were observed in human atrial and rabbit sinoatrial cells. In conclusion, mibefradil combines the voltage- and use-dependent effects of dihydropyridines and benzothiazepines on I-CaL. Inhibit ion of I-CaL, which has probably been underestimated before, may contribute to most of the cardiovascular effects of mibefradil.

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